Adult vitamin D deficiency leads to behavioural and brain neurochemical alterations in C57BL/6J and BALB/c mice

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Abstract

Epidemiological evidence suggests that low levels of vitamin D may predispose people to develop depression and cognitive impairment. While rodent studies have demonstrated that prenatal vitamin D deficiency is associated with altered brain development, there is a lack of research examining adult vitamin D (AVD) deficiency. The aim of this study was to examine the impact of AVD deficiency on behaviour and brain function in the mouse. Ten-week old male C57BL/6J and BALB/c mice were fed a control or vitamin D deficient diet for 10 weeks prior to, and during behavioural testing. We assessed a broad range of behavioural domains, excitatory and inhibitory neurotransmission in brain tissue, and, in separate groups of mice, locomotor response to D-amphetamine and MK-801. Overall, AVD deficiency resulted in hyperlocomotion in a novel open field and reduced GAD65/67 levels in brain tissue. AVD-deficient BALB/c mice had altered behaviour on the elevated plus maze, altered responses to heat, sound and shock, and decreased levels of glutamate and glutamine, and increased levels of GABA and glycine. By contrast C57BL/6 mice had a more subtle phenotype with no further behavioural changes but significant elevations in serine, homovanillic acid and 5-hydroxyindoleacetic acid. Although the behavioural phenotype of AVD did not seem to model a specific disorder, the overall reduction in GAD65/67 levels associated with AVD deficiency may be relevant to a number of neuropsychiatric conditions. This is the first study to show an association between AVD deficiency and prominent changes in behaviour and brain neurochemistry in the mouse.

1. Introduction

The role of vitamin D in adult brain function has attracted considerable attention in recent years [1]. Apart from the well-established role in calcium homeostasis, key features of the vitamin D system have been identified in the central nervous system. For example, the vitamin D receptor and the key enzyme required for its activation, 25-OH D_3 1α hydroxylase, has been identified in both neuronal and glial cells in the human brain [2]. Vitamin D has been linked to key neurotrophic agents, such as nerve growth factor and glial cell-derived neurotrophic factor [3,4], and animal experiments have suggested that the active form of the vitamin has 'neuroprotective' features. For example, pre-treatment with vitamin D attenuates the effects of various stressors, including 6-hydroxydopamine-induced neurotoxicity [5,6]. Moreover, vitamin D deficiency in adult rats has been shown to exacerbate stroke injuries and lead to more severe post-stroke behavioural impairments and this was accompanied by lower levels of the neuroprotective hormone, insulin-like growth factor 1 [7].

Clues from epidemiology suggest that developmental vitamin D (DVD) deficiency may be associated with an increased risk of schizophrenia [8] and there has been considerable focus on animal models that examine the impact of DVD deficiency on brain related outcomes [9-11]. For example, rats exposed to DVD deficiency have decreased expression of nerve growth factor [4]. They also have ventricular enlargement as neonates [9] a finding that persists through to adulthood [4]. DVD

neonates have alterations in catechol-O-methyl transferase expression and dopamine metabolism [12] and early alterations in dopamine ontogeny [13]. Links between dopamine and vitamin D have also been identified in adult animals. Vitamin D has been shown to promote the synthesis of tyrosine hydroxylase, the rate-limiting enzyme of dopamine synthesis and also to increase basal levels of dopamine within the central nervous system after localised injection of vitamin D_3 [6,14].

The research community has become more aware of the role of vitamin D in adult brain function and epidemiological studies have examined links with various neuropsychiatric disorders. For example, cross-sectional studies have reported significant associations between low concentrations of 25-OH D_3 in adults and various indicators of cognitive function, including memory and orientation [15-17], executive function [18,19], and diagnosis of dementia and Alzheimer's disease [20]. A recent prospective study [21] found that baseline vitamin D deficiency was associated with greater impairment of cognitive tasks in subsequent years. However, other studies have not found an association between vitamin D status and brain outcomes [22-24].

Studies on European populations have shown that second generation dark-skinned migrants (with a reduced ability to generate vitamin D from sunlight) have an increased risk of being diagnosed with schizophrenia compared to other immigrants and an even greater risk compared to the native born [25]. Vitamin D deficiency has been linked to a number of other neurological disorders including autism [26], seasonal affective disorder [27,28], and multiple sclerosis [29]. Cross-sectional studies have reported an association between vitamin D status and depressive symptoms [30]. A prospective study reported that women with low levels of vitamin D at baseline reported higher levels of depressive symptoms 3 and 6 years later [31].

To date, there has only been one study to investigate the impact of AVD deficiency on brain function and behaviour in rodents [32]. However, in that study the Sprague-Dawley rats were fed a vitamin D deficient diet from weaning and they had reduced body weight compared to controls, which suggests that musculoskeletal problems may have confounded interpretation of behavioural outcomes in the study. AVD deficiency has previously been associated with altered catecholamine metabolism in the cortex of Sprague-Dawley rats, with alterations in the levels of noradrenaline and dihydroxyphenylacetic acid, a breakdown product of dopamine [33]. However, there was no behavioural data reported. Thus, to our knowledge there are no published data on the impact of AVD deficiency on mouse behaviour or brain function and thus it is difficult to predict how this may impact on different behavioural domains or brain neurochemistry in mice.

The aim of this study was to establish a model of AVD deficiency in two strains of mice and to examine a broad range of behavioural tests and selected neurochemical outcomes within the brain. BALB/c and C57BL/6J strains were chosen for this study because they are inbred strains that are commonly used in biomedical research, and show markedly different behavioural phenotypes [34]. The overarching aim of this work was to establish a model of adult vitamin D deficiency, rather than model a single neuropsychiatric disorder. Indeed, a recent review highlighted the fact that vitamin D deficiency is associated with a range of neuropsychiatric outcomes [11]. As such we used a multi-tiered behavioural test battery to broadly assess the behavioural domains of locomotion, exploration, anxiety, social behaviour, learned helplessness, sensorimotor gating, associative learning, and nociception, as well as responses to the psychomimetic agents D-amphetamine and MK-801. These behavioural tasks are relevant to a number of neuropsychiatric disorders including

pyschosis, schizophrenia, anxiety and depression. Neurochemical profiles were analysed in drug-naïve mice in order to explore the effect of AVD deficiency on levels of catecholamines and amino acids, as well as selected enzymes involved with key neurotransmitters.

2. Materials and Methods

2.1 Animals and housing

A total of 148 male mice were used in this study (79 x C57BL/6J and 69 x BALB/c). Ten week old C57BL/6J and BALB/c mice (Animal Resources Centre, Canning Vale, WA, Australia) were obtained and housed in groups of 4 in individually ventilated cages (Techniplast, VA, Italy), with corn cob bedding (Shepherd Specialty Papers, Inc., TN, USA) at the Eskitis Animal House Facility, Griffith University. The mice were assigned to either a control diet (Standard AIN93G Rodent diet with 1,000 IU vitamin D_3/kg, Specialty Feeds, WA, Australia) or a vitamin D-deficient diet (Vitamin D_3 Deficient AIN93G Rodent diet, Specialty Feeds, WA, Australia) for a minimum of 10 weeks prior to the start of behavioural testing; and for the entire duration of the experimental procedures. The mice were maintained on a 12-hour light-dark cycle (lights on at 07:00 h) with *ad libitum* access to food and water. They were housed under incandescent lighting free from UVB radiation. All experimental work was performed with approval from the Griffith University Animal Ethics Committee, under the guidelines of the National Health and Medical Research Council of Australia.

2.2 Procedure

Behavioural phenotyping began when the on postnatal day 140 (P140). Mice were exposed to a variety of tests assessing various behavioural domains. The tests were

performed on separate days and in the following order (approximate age of mice at each test); open field (P140), elevated plus maze (P168), holeboard (P169), light/dark test (P170), forced swim test (P171), prepulse inhibition of the acoustic startle response (P175), active avoidance (P182), social interaction (P189), hot plate test (P196). Sixteen C57BL/6J mice (*n* = 8/diet) and sixteen BALB/c mice (*n* = 8/diet) were subjected to the behavioural test battery, with additional mice from each strain tested in the novel open field (n = 28/diet/strain), ASR, and PPI of ASR (n = 20/diet/strain). Behaviours were recorded by two methods, either by video recording software (Miglia TV, Hertfordshire, UK) or a USB webcam and recording software (Media Player) and analysed by computer tracking software (Ethovision, Noldus, Wageningen, The Netherlands). All apparatus was cleaned before and after each mouse with 80% ethanol.

2.2.1 Open Field

The open field test was used to measure baseline locomotion [35]. The open field apparatus was an opaque box (28 cm high x 30 cm x 30 cm), in which the mice were placed in the centre of the arena. The test lasted for 30 min, during which time both total distance travelled and distance travelled over time were analysed to assess locomotion. Locomotion was assessed in a novel environment, and also in a familiar environment, with a subset of mice (n=9-10 per group) re-exposed to the same open field arena 7 weeks later.

2.2.2 Elevated plus maze

The elevated plus maze was used to measure anxiety-related behaviour [36]. The elevated plus maze was made of opaque grey acrylic, and was attached to a stand, raising it 50 cm off the ground. It was comprised of four arms (each 5 cm x 30 cm)

radiating out from a centre platform (5 cm x 5 cm) in the shape of a plus. Two opposing arms had sides (closed arms) and the other two did not (open arms). Mice were placed on the centre platform, facing out towards one of the open arms, with the test lasting for 10 min. The main parameter tested was the time spent on the open arms compared to the time spent on the closed arms. The total distance travelled in the 10 min test was also analysed.

2.2.3 Holeboard Test

The holeboard test was used to measure exploration based on the frequency of head-dipping into small holes situated in the floor of an open field [37]. A white acrylic base containing four evenly spaced holes (2.5 cm diameter), was added to the bottom of a black box (30 cm high x 30 cm x 30 cm) to create the holeboard. The mice were initially placed along the edge of the wall and the number of head-dips in a 10 min period was analysed, along with the total distance travelled.

2.2.4 Light/dark test

The light/dark test was used in the behavioural test battery to aid in the analysis of anxiety levels of the mice [38]. The two-chambered light/dark apparatus consisted of an open black box (30 cm high x 30 cm x 30 cm), with half of the box containing a black insert (15 cm high x 30 cm x 15 cm) creating the dark chamber. The insert had a small rounded open doorway in the centre leading to the light chamber. The mice were placed in the doorway with their head inside the dark chamber and two main parameters were analysed. The time for the mouse to emerge from the dark chamber into the light and the percentage of time the mouse spent in the light chamber in a 10 min period.

2.2.5 Forced swim test

The forced swim test was used to measure learned helplessness [39]. The apparatus used in the forced swim test was a clear round container (20 cm high x 13 cm diameter) with a column of water (16 cm deep) maintained at 25 °C, from which the mouse could not escape. The percentage of time the mouse spent immobile in each one min time bin, over a 10 min period was the parameter analysed.

2.2.6 Prepulse inhibition of the acoustic startle response

PPI of the ASR was used to measure sensorimotor gating [40] using startle chambers (SR-Lab, San Diego Instruments, CA, USA), which consisted of a Plexiglass cylinder (5 cm diameter x 12 cm long) mounted on an elevated Plexiglass base within a dark chamber. A speaker situated 24 cm above the cylinder was used to provide background noise within the chamber set to 70 dB as well as the acoustic pulses of white noise throughout the testing.

Testing began with an acclimatisation period of 300 s of 70 dB background noise. The mice then underwent a total of 130 trials (26 different blocks of 5 trials). To assess within-trial habituation, startling pulses of 110 dB were presented at the start (post acclimatisation), middle and end of the testing. The mice were exposed to a range of pulse intensities (80, 90, 100, 110 and 120 dB) to measure ASR and a range of prepulse to pulse intervals (8, 16, 32, 64, 128 and 256 ms) before a 120 dB pulse to measure PPI. The median values for each block of 5 trials were used for analysis, with PPI being calculated with the formula: %PPI = [(startle amplitude of ASR trial - startle amplitude on prepulse trial)/startle amplitude of ASR trial] x 100.

2.2.7 Active avoidance

The active avoidance test was used to measure associative learning, using a conditioned avoidance response [41]. The active avoidance chambers (Gemini, CA, USA) consisted of two compartments (18 cm high x 26 cm x 21 cm) separated by a guillotine door (10 cm high x 7 cm) above a barrier. Testing began with a 300 s acclimatisation period followed by 80 trials. Each trial was comprised of a 4 s conditioned stimulus, followed by a 16 s unconditioned stimulus. The conditioned stimulus consisted of a tone, cue light and the opening of the door and the unconditioned stimulus consisted of a 0.4 mA shock delivered to the floor. The door between the chambers remained open during the trial until the mouse moved through it to the other compartment. Each trial was separated by a 20 s inter-trial interval with no stimulus, with the door between compartments closed.

Active avoidance for each mouse was analysed over 3 consecutive days. The first two days followed the above method for acquisition and retention; with the third day, a test of extinction, in which the unconditioned stimulus was removed. Each trial still began with the 4 s conditioned stimulus, but was followed by 16 s with no stimulus, in order to test the mouse's ability to learn a new response to the original conditioned stimulus.

2.2.8 Social interaction test

The social interaction test was used to measure the behavioural responses of two unfamiliar, diet and weight matched mice to each other [42]. The test was conducted in an open field arena and behaviours were analysed over a 10 min period. A variety of behaviours were assessed including sniffing, self-grooming, allo-grooming, following and rearing.

2.2.9 Hot plate test

The hot plate test was used to measure a response to noxious stimuli [43]. The hot plate (Harvard Apparatus, Ltd., Kent, England) was maintained at 53 °C, with the mice contained on the hot plate within a clear Perspex cylinder (27 cm high x 20 cm diameter). The test consisted of three trials, of a maximum length of 30 s. The parameter measured was the latency to lick hind paw or to jump with both feet, in an attempt to escape. The mouse was removed immediately after this was achieved. If this was not achieved by the end of the 30 s, the mouse was removed and the trial ended. The inter-trial period was 60 s.

2.3 Brain Neurochemistry

2.3.1 Tissue Collection

Body weight was monitored on a fortnightly basis throughout the study for all mice. At the time on euthanasia, body measurements were taken from both strains of mice, for body length (from tip to snout to base of tail) and tail (from base to tip). Brains were collected from drug-naïve mice subjected to the behavioural test battery. Mice were euthanized by carbon dioxide followed by decapitation. The whole brain was removed and weighed. Using free-hand dissection, each brain was sectioned into left and right cerebrum and hindbrain, with olfactory bulbs removed. Tissue was frozen on dry ice and stored at -80 °C until further processing.

2.3.2 High performance liquid chromatography and analysis

Catecholamines and amino acids from brain tissue were measured by high performance liquid chromatography with electrochemical and fluorescent detection using a standard protocol. Data was stored and processed with ChemStation software (B1.03.02, Agilent Technologies, Inc., CA, USA). Data was quantified by

calculating peak-area ratios of each compound compared to deoxyepinephrine (catecholamine internal standard) or homoserine (amino acid internal standard) and corrected for dilution. Identity of each compound was determined by retention time and the final amount was expressed as nanogram per gram (ng/g) wet tissue.

2.3.3 Protein analysis

Brain tissue was analysed for protein content using western analysis. Total protein was collected by sonication in 20 mM Hepes, 1 mM EDTA lysis buffer (pH 7.4) containing 1% Triton and 1X Protease Inhibitor cocktail (Sigma). Following centrifugation, protein concentrations in the supernatant were determined using a BCA protein assay (Pierce, Rockford, IL, USA). Between 15 and 30 μg of protein was loaded for all samples dependent on protein to be analysed. Proteins were separated by standard polyacrylamide gel electrophoresis techniques on 4-12% Bis-Tris SDS-PAGE gels. Separated proteins were transferred to Immobilon-FL membranes (Millipore, Bedford, MA, USA) at 0.4 A for 2 h at room temperature.

Membranes were blocked in Lycor Odyssey Blocking buffer (Millenium Science, VIC, Australia) for 1 h and incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C in Lycor Odyssey blocking buffer. The primary antibody dilutions were as follows: catechol-O-methyl transferase, monoclonal mouse anti-mouse antibody (BD Biosciences, San Jose, CA, USA) 1:2000; monoamine oxidase A, polyclonal rabbit anti-human antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:2000; glutamine synthetase, polyclonal rabbit anti-human antibody (Abcam, Cambridge, UK) 1:12,000; GAD 65/67, polyclonal rabbit anti-human antibody (Sigma-Aldrich, St Louis, MO, USA) 1:150,000; and the normalising gene

glyceraldehyde-3-phosphate dehydrogenase, monoclonal mouse anti-rabbit antibody (Millipore, Bedford, MA, USA) 1:100,000).

The membranes were then washed in phosphate buffered saline with 1% Tween 20 for 3x 10 min and exposed to secondary antibodies conjugated with IRDyes for 1 h at room temperature. The IRDye 800CW-polyclonal goat anti-rabbit antibody (Millenium Science, VIC, Australia) was diluted 1:15,000 for binding to monoamine oxidase A antibodies and 1:30,000 for binding to GAD65/67 antibodies. The IRDye 680-polyclonal goat anti-mouse antibody (Millenium Science, VIC, Australia) was diluted 1:20,000. The membranes were again washed in phosphate buffered saline with 1% Tween 20 for 3x 10 min and the protein bands visualized on a Licor Odyssey CLx scanner. Amount of proteins were normalised against glyceraldehyde-3-phosphate dehydrogenase.

2.4 Behavioural Pharmacology

The open field arena was used to assess behaviour in response to two psychomimetic drugs in separate groups of mice. Mice were habituated to the open field for 30 min before receiving an intra-peritoneal injection of a 4 ml/kg 0.9 % saline solution. They were placed back in the open field for a further 30 min following this injection (Data not shown). They then received an intra-peritoneal injection of a 4 ml/kg solution of either D-amphetamine (control *n=9,* AVD *n=10)* or MK-801 (control *n=8,* AVD *n=8)*. They were placed back in the open field and their behaviour was recorded for up to 210 min.

2.4.1 Psychomimetic Drugs

D-amphetamine (Sigma, MO, USA) was diluted to a concentration of 1.25 mg/ml in

0.9 % saline solution and aliquots were stored at -20 °C. Mice were given an injection dose of 5 mg/kg. MK-801 (Sigma, MO, USA) was diluted to a concentration of 0.125 mg/ml in 0.9 % saline solution and aliquots were stored at -20 °C. Mice were given an injection dose of 0.5 mg/kg. The drug dosage used was based on previous studies showing a high locomotor response with little stereotypic or ataxic behaviours [44,45].

2.5 Blood chemistry

At the completion of experimental procedures, a terminal blood sample was taken from each mouse via cardiac puncture. The levels of 25-OH D_3 was measured in serum samples using liquid chromatography-tandem mass spectrometry (Sciex Instruments, ON, Canada) on a 4000 QTrap API AB mass spectrometer [46]. BALB/c control: 32.0 ± 1.7 nM, AVD-deficient: 2.9 ± 0.2 nM, and C57BL/6J control: 65.3± 2.3 nM, AVD-deficient: 2.9 ± 0.1 nM.

Calcium levels (*n*=17-19) were measured in serum samples using the QuantiChrom Assay Kit using quantitative colormetric determination (BioAssay Systems, CA, USA) and were not significantly different between groups. BALB/c control: 2.33 ± 0.15 mM, AVD-deficient: 2.20 ± 0.09 mM, C57BL/6J control: 2.28 ± 0.09 mM, AVD-deficient: 2.13 ± 0.10 mM).

2.6 Statistical analysis

Results were analysed for statistical significance using SPSS (version 17.0) software. All data were analysed for the main effects of Diet (control or AVD-deficient) and Strain (C57BL/6J or BALB/c) using analysis of variance (ANOVA) or, where appropriate, repeated measures ANOVA. Mann-whitney U test was used to

analyse non-parametric data. Significant differences ($p<0.05$) were followed up with post-hoc *t*-tests.

3. Results

3.1.1 Open Field

The locomotor response was measured both in a novel environment and in a familiar environment. Overall, AVD-deficient mice from both strains exhibited increased locomotion compared to the controls in a novel environment ($F_{1,108}$ = 11.18, p = 0.001) (Fig.1a and b). There was a significant effect of Diet in both BALB/c ($F_{1,54}$ = 4.13, p = 0.047) and C57BL/6J mice ($F_{1,54}$ = 9.11, p = 0.004). Repeated measures ANOVA indicated that mice were significantly less active when subsequently tested under familiar conditions, compared with activity levels under novel conditions ($F_{1,34}$ =21.1, $p < 0.001$). However, there was a significant Strain x Diet interaction on the locomotor response in the familiar environment ($F_{1,34}$ =6.5, $p < 0.05$); the AVD-deficient BALB/c mice showed a significant reduction in locomotion compared with control BALB/c mice ($F_{1,17}$ = 8.66, p = 0.009), while there was no significant effect of Diet in the C57BL/6J mice ($F_{1,17}$ = 1.42, p = 0.250) (Fig.1c and d).

<<Insert Fig. 1 about here>>

3.1.2 Elevated plus maze

On the elevated plus maze, all mice entered the closed arms and all but two control mice entered the open arms at least once. There was a significant effect of Diet on the time spent on the open arms for the BALB/c mice ($F_{1,14}$ = 14.57, p = 0.002). Compared to controls, the BALB/c AVD-deficient mice spent a significantly longer

time on the open arms (Fig. 2a and b). There was no significant effect of Diet for the C57BL/6J mice ($F_{1,14}$ = 0.43, p = 0.839). There was a significant main effect of Strain ($F_{1,28}$ = 10.07, p = 0.004) on the distance travelled while on the elevated plus maze with C57BL/6J mice travelling further than the BALB/c mice, however, there was no significant effect of Diet on total distance travelled.

<<Insert Fig. 2 about here>>

3.1.3 Holeboard test

In the holeboard test, all mice poked their noses into the holes. There was a significant main effect of Strain on the frequency of head-dipping ($F_{1,28}$ = 5.12, p = 0.032) as well as on the latency to groom ($F_{1,28}$ = 4.26, p = 0.048) and the total distance travelled ($F_{1,28}$ = 20.71, p = <0.001). However, there was no significant effect of Diet on any measure on the holeboard test.

3.1.4 Light/dark test

All mice entered the light chamber at least once during the test. There was no significant effect of Diet or Strain on the time to emerge, however, there was a significant effect of Strain on the frequency to enter the light chamber ($F_{1,28}$ = 22.89, p <0.001). There was no effect of Diet on any measure on the light/dark test.

3.1.5 Forced swim test

All mice became increasingly immobile over the course of the forced swim test. There was no significant difference in the time spent immobile between diet groups from either strain. However, there was a significant effect of Strain ($F_{1,28}$ = 417.48, p

<0.001) with the BALB/c mice spending a greater amount of time immobile than the C57BL/6J mice.

3.1.6 Prepulse inhibition of the acoustic startle response

All mice responded to increasing pulse amplitude with increased startle response. There was a significant interaction of Diet x Strain ($F_{1,75}$ = 5.93, p = 0.017). Although there was no significant effect of Diet on the ASR of C57BL/6J mice ($F_{1,38}$ = 0.03, p = 0.874), there was a significant effect of Diet for the BALB/c mice ($F_{1,37}$ = 23.52, p <0.001) with AVD-deficient mice showing an enhanced startle response compared to controls (Fig. 2c and d). There was no significant main effect of Diet ($F_{1,75}$ = 0.08, p = 0.785) or Strain ($F_{1,75}$ = 0.55, p = 0.459) on PPI scores.

3.1.7 Active avoidance

Mice of each strain responded quite differently to the test of avoidance learning and some of the BALB/c mice had difficulty learning the avoidance paradigm all together. There was a significant Diet x Strain interaction for acquisition of learning (Day 1) ($F_{1,28}$ = 4.63, p = 0.040). There was no significant effect of Diet on the latency to escape in C57BL/6J mice but there was a significant effect of Diet on the latency to escape for BALB/c mice during acquisition ($F_{1,14}$ = 6.44, p = 0.024) (Fig. 3a and b). This difference did not reach significance on Day 2, for retention ($F_{1,14}$ = 4.12, p = 0.062) or on Day 3, for the extinction trial ($F_{1,30}$ = 1.07, p = 0.310).

There was no significant effect of Diet for the C57BL/6J mice on any of the response scores (avoid, escape or no response) on Day 1 or over the 3-day test. There was a significant effect of Diet ($F_{1,14}$ = 6.62, p = 0.022) for the BALB/c mice on the no response score on Day 1 of the test; the AVD-deficient mice made fewer no response

scores than the controls. The effect of Diet on the no response score over the 3-day test did not reach significance ($F_{1,30}$ = 4.05, p = 0.053).

3.1.8 Social interaction test

In the social interaction test there was no significant effect of Diet on either strain for any measure analysed. There were significant effects of Strain for following ($F_{1,25}$ = 29.82, p <0.001) and rearing ($F_{1,25}$ = 60.63, p <0.001) with BALB/c mice having reduced following and rearing compared to the C57BL/6J mice.

3.1.9 Hot plate test

On the first trial of the hot plate test, there was a significant main effect of Strain ($F_{1,28}$ = 51.02, p <0.001) but not of Diet: the C57BL/6J mice had a shorter latency to lick the hindpaw than the BALB/c mice. When analysing the BALB/c mice separately, there was a significant effect of Diet on the first trial (U_{14} = 16, p = 0.0325) with all the control BALB/c mice being removed from the hot plate at the end of the trial (30 s), because none of them licked their hind paw in response to the heat stimulus.

When analysing the average response over three trials, there was a significant main effect of Strain ($F_{1,28}$ = 72.72, p <0.001) and also a significant main effect of Diet ($F_{1,28}$ = 10.12, p = 0.004) with no Strain x Diet interaction. When analysing the strains separately, there was a significant effect of Diet ($F_{1,14}$ = 7.86, p = 0.014) in the BALB/c strain but not in the C57BL/6J mice ($F_{1,14}$ = 3.46, p = 0.084), the BALB/c AVD-deficient mice had a shorter latency to lick the hind paw compared to the control BALB/c mice (Fig. 3c and d).

<<Insert Fig. 3 about here>>

3.2 Brain Neurochemistry

Analysis of the neurotransmitter and metabolite levels from brain tissue indicated a significant effect of Strain on levels of noradrenaline ($F_{1,28}$ = 27.05, p = <0.001), 5-hydroxyindoleacetic acid ($F_{1,28}$ = 22.62, p = <0.001), 3-methoxytyramine ($F_{1,28}$ = 12.60, p = 0.001), serine ($F_{1,28}$ = 16.45, p = <0.001), glycine ($F_{1,28}$ = 200.54, p = <0.001), tyrosine ($F_{1,28}$ = 12.65, p = 0.001), GABA ($F_{1,28}$ = 65.02, p = <0.001), and methionine ($F_{1,28}$ = 6.15, p = 0.019).

When analysing the BALB/c strain separately, there was a significant effect of Diet on the levels of glutamine ($F_{1,14}$ = 4.98, p = 0.043), glutamate ($F_{1,14}$ = 6.69, p = 0.022), glycine ($F_{1,14}$ = 6.24, p = 0.026) and GABA ($F_{1,14}$ = 5.90, p = 0.029). Glutamine and glutamate levels were significantly lower in the AVD-deficient mice, whereas levels of glycine, lysine, and GABA were significantly higher in the AVD-deficient mice (Table 1).

There was a significant effect of Diet on the levels of 5-hydroxyindoleacetic acid ($F_{1,14}$ = 12.97, p = 0.003), homovanillic acid ($F_{1,14}$ = 4.69, p = 0.048), and serine ($F_{1,14}$ = 28.04, p = 0.000) in C57BL/6J mice. The levels of all three were higher in the AVD-deficient mice (Table 1). The ratio of 5-hydroxytryptamine to 5-hydroxyindoleacetic acid was significantly altered ($F_{1,14}$ = 9.16, p = 0.009) as was the ratio of dopamine to homovanillic acid ($F_{1,14}$ = 8.34, p = 0.012). A summary of all the significant effects of Diet are found in Table 2.

<<Insert Table 1 about here>>

3.3 Protein analysis

GAD65/67 content showed a significant main effect of Diet ($F_{1,28}$ = 6.41, p = 0.017) and of Strain ($F_{1,28}$ = 38.17, p < 0.001) with no Strain x Diet interaction. The levels of GAD65/67 were lower in the AVD-deficient mice from both strains when compared to controls (Fig. 4).

There was no significant effect of Diet on levels of catechol-O-methyl transferase ($F_{1,27}$ = 0.12, p = 0.734), monoamine oxidase A ($F_{1,27}$ = 3.25, p = 0.083) or glutamine synthetase ($F_{1,28}$ = 0.38, p = 0.544) in the brain of either strain (data not shown).

<<Insert Fig. 4 about here>>

<<Insert Table 2 about here>>

3.4 Behavioural Pharmacology

As expected, locomotion was greatly increased by acute treatment with either amphetamine or MK-801. There was no significant main effect of Diet on the locomotor response to D-amphetamine ($F_{1,34}$ = 0.002, p = 0.965) but there was a significant effect of Strain ($F_{1,34}$ = 17.39, p = <0.001), with the C57BL/6J mice showing greater amphetamine-induced locomotion than the BALB/c mice (Fig. 5a and b). There was no significant main effect of Diet ($F_{1,28}$ = 0.99, p = 0.329) or Strain ($F_{1,28}$ = 2.65, p = 0.115) on the locomotor response to MK-801 (Fig. 5c and d).

<<Insert Fig. 5 about here>>

4. Discussion

The main outcomes of this study were that vitamin D deficiency in adult mice enhanced locomotion and produced a small reduction in the enzymes involved in GABA synthesis (GAD65/67). However, many other behavioural and neurochemical alterations were dependent on the background strain. The AVD-deficient BALB/c mice displayed altered behaviour on the elevated plus maze, and an enhanced response to aversive stimuli that included shock, heat and sound. This was accompanied by alterations in amino acid metabolism within the brain. AVD-deficient C57BL/6J mice had a more subtle behavioural phenotype, and a neurochemical profile with changes in dopamine and 5-hydroxytryptamine turnover.

Spontaneous hyperlocomotion in a novel open field has been consistently observed in other rodent models of vitamin D deficiency, although these have been prenatal exposures. For example, 129/SvJ DVD-deficient mice exhibited spontaneous hyperlocomotion in the open field, and 129/SvJ and C57BL/6J DVD-deficient mice were hyper-explorative on the hole-board test [40]. Adult DVD deficient rats are more active than controls in novel environments [47,48], show an increased psychomimetic-induced hyperlocomotion and an enhanced startle response after MK-801 injection [49,50]. This suggests that the absence of vitamin D may lead to alterations in similar neural circuits in both the developing and adult brain.

With respect to schizophrenia, the AVD mice did not replicate behavioural features typically seen in other animal models of schizophrenia. For example, two prominent features include deficits in PPI [51] and enhanced locomotor response to amphetamine [49]. These features are both based on alterations in dopamine metabolism, with the dopaminergic system being strongly implicated in the development of schizophrenia [52]. Moreover, although DVD-deficient rats show enhanced MK-801 induced locomotion [48,50] this was not seen in DVD-deficient

129/SvJ or C57BL/6J mice [45] or in AVD-deficient BALB/c or C57BL/6J mice in the current study.

However, the AVD-deficient BALB/c mice had alterations in glutamate and glutamine levels in brain tissue, which may indicate a disruption in glutamatergic neurotransmission. There is mounting evidence to suggest that NMDA receptor dysfunction may contribute to schizophrenia symptoms, particularly those that are not relieved by antipsychotics. This dysfunction may be related more to the negative and cognitive symptoms of schizophrenia [53]. It has also been suggested that non-NMDA receptor dysregulation may be involved in schizophrenia [54]. While the alterations in glutamate metabolism seen in the AVD-deficient BALB/C mice did not translate into known behavioural features of schizophrenia, this does not rule out the relevance these mice might have in schizophrenia research and for other neurological disorders, such as anxiety.

The underlying neurobiological mechanisms of the behaviour of AVD deficient mice remain to be clarified. Despite a consistent decrease in GAD65/67 levels in both strains of mice used in this study, there was little overlap between strains for changes seen in brain neurochemistry. The small but significant reduction in GAD65/67 levels may be highly relevant to psychiatric conditions in which altered GABAergic neurotransmission has been implicated, including schizophrenia [55], autism [56], anxiety [57], depression [58], bipolar disorder [57,59] and obsessive compulsive disorder [57,60]. For example, reductions in GAD67 in parietal cortex and GAD65 in cerebellum were shown in post mortem brain tissue from autistic patients when compared to controls [61]. Another study reported global reductions in levels of GAD65/67 in the cerebella of subjects with schizophrenia, bipolar disorder and major depression [59]. Despite reports of small global reductions in GAD 65/67

these changes were shown to be accounted for by alterations in distinct subpopulations of neurons, including the larger-sized GABAergic dentate cells within the cerebellum [62]. In the current study subtle variations in specific brain regions or cell populations could not be detected as all analyses were performed on whole brain homogenates. Future studies would need to investigate localised changes in defined cell populations.

The two strains used in this study differed on a range of physiological parameters, behavioural responses, and drug-induced behaviours, as well as in levels of several neurochemicals. The non-emotional, high locomotor C57Bl/6 strain showed very little effect of AVD deficiency except spontaneous hyperlocomotion, while the highly emotional, neophobic BALB/c strain showed significant effects of AVD deficiency, particularly involving limbic system functions. One of the major differences found between BALB/c and C57Bl/6 mice is the strong defensive behaviour the BALB/c mice show in response to unfamiliar places (neophobia). This is proposed as 'trait' anxiety, a stable characteristic of behaviour. Administration of benzodiazepines is able to abolish neophobia in BALB/c mice, whereas several other anxiolytic compounds cannot. Benzodiazepines are also devoid of anxiolytic effects in C57Bl/6 mice [63]. The BALB/c strain shows a significant reduction in benzodiazepine receptor density compared to the C57Bl/6 strain [64]. This may at least in part explain the genetically determined predisposition to anxiety seen in the BALB/c mice.

There was a 50% reduction in 25-OH D_3 levels in control BALB/c mice compared to control C57BL/6 mice. Although AVD resulted in 25-OH D_3 levels at the lower limit of detection, we suggest that there may be underlying differences in vitamin D metabolism between the two strains. For example, BALB/c mice have a 17-fold reduction in the levels of 1αOHase within the kidney compared to C57BL/6J mice

[65], which reduces the availability of active 1,25 dihydroxyvitamin D_3 in the BALB/c strain. Moreover, wide variation in basal 25(OH)D levels have been reported in 18 inbred mouse strains [66], the highest values were obtained in C57BL/6J (62.5 nmol/L), with reduced amounts in all other strains examined, for example EVB/J (55 nmol/L) C3H/HeJ (43 nmol/L), BALB/cJ (42 nmol/L), DBA/2J (37 nmol/L), to the lowest SJL/J (35 nmol/L). These values are consistent with those reported in our study; C57Bl/6J (65.3 nmol/L) and BALB/cJ (32.0 nmol/L). Therefore, the BALB/c strain may be genetically vulnerable to vitamin D deficiency, which could explain why they show a greater effect of the vitamin D deficiency both on behaviour and brain function. However, there are, as yet, no systematic reports in the literature correlating behavioural endophenotypes and basal vitamin D levels in multiple inbred mouse strains. It may be that animals with chronic insufficiency (such as the BALB/c strain) are more prone to develop behavioural disorders if they are exposed to a period of vitamin D deficiency. These studies may be a valuable area of future research.

The biological consequences of relatively small changes in neurotransmitter levels remains to be established. However, we found significant strain differences in the effect of AVD deficiency on catecholamine, indolamine and amino acid levels, with a predominantly catecholamine/indolamine phenotype in C57BL/6 mice and a predominantly amino acid phenotype in BALB/c mice. There is significant variation between C57BL/6 and BALB/c mice on metabolic protein levels in brain tissue. For example, differences were shown for nucleic acid, amino acid and carbohydrate metabolism in proteomic analysis of hippocampal tissue from these strains of mice [67]. Strain dependent regulation of amino acid metabolism could explain why AVD-deficiency had a greater impact on GABAergic and glutamatergic neurotransmitter

systems in BALB/c mice. With respect to indolamine levels (5-HT and 5-HIAA) C57BL/6 mice have approximately 50% higher rates of 5-HT synthesis than BALB/c mice because of a single nucleotide polymorphism in the mTph2 gene (C1473G) [68], which may lead to a greater vulnerability in AVD-deficient C57BL/6 mice when compared with BALB/c mice.

The AVD-deficient BALB/c mice had a small increase in the levels of GABA and glycine, and decreased levels of glutamate and glutamine in brain tissue. GABA is the major inhibitory neurotransmitter in the brain and it is synthesised from glutamate by two isoforms of GAD; GAD65 and GAD67. Reduced levels of GAD65/67 are typically associated with a reduction in GABA synthesis. However, we observed an increase in GABA levels in AVD-deficient BALB/c mice and, despite a similar reduction in GAD65/67 levels in C57BL/6 mice, there were no significant changes to either glutamate or GABA levels in this strain. The mechanism/s by which vitamin D deficiency could lead to increases in GABA are unknown. However, recent studies in developing zebrafish indicate that the active metabolite of vitamin D affects a wide range of genes, including those involved with amino acid metabolism [69], although similar studies have not been carried out in rodents. It was shown that GAD65 KO mice maintain normal levels of GABA content in brain [70] and the overall reduction in GAD enzymes may signify a reduced conversion of glutamate to GABA, but differences in biochemical alterations following a reduction in GAD65/67 may vary between BALB/c and C57BL/6 mice.

Positive modulators of GABAergic neurotransmission that function to increase GABA brain levels produce anxiolytic effects in rodent models of anxiety [71]. Chlordiazepoxide is an anxiolytic, GABA-enhancing agent, which, when given to BALB/c mice, produces several of the behavioural outcomes seen in this study.

Chlordiazepoxide administration in mice can lead to an increase in the time spent on the open arms of the elevated plus maze and lead to enhanced avoidance responses during active avoidance [72,73]. Repeated daily doses of chlordiazepoxide have also been shown to increase locomotion in the open field in BALB/c mice [74]. Although we did not measure functional changes in GABA receptors, it may be that the increased levels of GABA observed here were sufficient to produce the behavioural phenotype. It is also possible that the neurochemical changes could either be a direct consequence of vitamin D deficiency, or indirectly via altered baseline behaviour, such as altered locomotion in the home cage.

There is little convergent evidence from three separate tests (EPM, holeboard, light/dark test) to suggest that AVD BALB/c mice had an anxiolytic phenotype. By contrast the evidence would suggest that the BALB/c AVD mice are more reactive, with enhanced open arm time on the EPM, greater ASR response, reduced latency to escape a footshock in the active avoidance, and reduced latency in the hot plate test. BALB/c mice typically freeze in response to aversive stimuli. The control BALB/c mice had difficulty responding in the active avoidance task. Instead of moving into the non-shocked compartment after the presentation of cues or foot-shock, they froze in place, unable to move. The AVD-deficient BALB/c mice seemed able to overcome their natural freezing instinct to react by moving into the non-shocked compartment on presentation of the stimulus, shortening their latency to escape over the length of the test when compared to controls. An increase in pain sensitivity could enhance the response to foot shock during avoidance learning and quicken their response in the hot plate test.

We did not compare directly the effects of critical periods of vitamin D deficiency (i.e. gestational versus adult) in the current study. Data from developmental studies

indicates that a number of different mechanisms may exist by which vitamin D impacts both during brain development and on adult, from altered proliferation, apoptosis or mitosis [11]. There are no data on developmental vitamin D deficiency in BALB/c mice. We have published data to show that DVD C57BL/6 mice also show hyperlocomotion (Harms et al., 2008) and this would suggest that there may be a similar impact on the developing and adult brain. Comparisons between developmental and adult vitamin D deficiency in BALB/c mice would need to be carried out to address this question directly. In addition, the current findings were from male mice and these may not be generalizable to female mice and so these studies would also need to be performed.

The results from the current study show that adult vitamin D deficiency has effects on behaviour and brain function in two inbred mouse strains. We found alterations consistent across both strains (hyperlocomotion and reduced GAD65/67) with many being altered only in the BALB/c strain, which was more susceptible to AVD than C57Bl/6. This suggests that our findings are not generaliseable to all mouse strains, although this is likely to be the case for many manipulations, for example environmental enrichment has strain specific effects on behaviour [75]. The current findings suggest that both excitatory and inhibitory neurotransmitter systems are affected by vitamin D deficiency in adult mice, which may be further exacerbated by the background strain or by detrimental environmental exposures or genomic instability. With vitamin D deficiency becoming increasingly recognised throughout the world [76,77], it is important to determine to what extent vitamin D deficiency contributes to adverse brain outcomes in light of the fact that supplementation is readily available. In particular, the findings seen in the AVD-deficient BALB/c mice

may provide a suitable tool for future investigations into the effects of vitamin D deficiency on the adult brain.

Figure 1. Behaviour in control and AVD-deficient C57BL/6J (a and c) and control and AVD-deficient BALB/c (b and d) mice in the novel open field (n=28 per group a and b) andin the familiar open field (n=9-10 per group, c and d). In the novel open field arena, the AVD-deficient mice from both the C57BL/6J and BALB/c strains showed significantly increased locomotion compared to controls. While, in the familiar open field the BALB/c AVD-deficient mice showed a significant reduction in locomotion when compared to controls, with no significant change in the C57BL/6J mice. $p<0.05$ (*), $p<0.01$ (**).

Figure 2. Behaviour in control and AVD-deficient C57BL/6J (a and c) and control and AVD-deficient BALB/c (b and d) mice on the elevated plus maze (n=8 per group, a and b) and acoustic startle response (n=20 per group, c and d). On the elevated plus maze, the BALB/c AVD-deficient mice spent significantly more time on the open arms when compared to control mice. In the acoustic startle response, BALB/c AVD-deficient mice had a significantly greater startle response than the control mice at pulse intensities of 90, 100, 110 and 120 dB. $p<0.01$ (**).

Figure 3. Behaviour in control and AVD-deficient C57BL/6J (a and c) and control and AVD-deficient BALB/c (b and d) mice in the test of active avoidance (a and b), and on the hot plate test (c and d). In the test of active avoidance, the BALB/c AVD-deficient mice had a reduced latency to escape on Day 1 during acquisition, when compared to controls. On the hot plate test, the BALB/c AVD-deficient mice had a significantly shorter latency to link the hind paw when compared to the control mice, averaged over the 3 trials. $p<0.05$ (*), n=8 per group.

Figure 4. Western Blots for GAD65/67 in control and AVD-deficient C57BL/6J (a) and control and AVD-deficient BALB/c (b) mice. There was a significant reduction in GAD65/67 for AVD-deficient mice compared to controls. Significance is noted as follows: <0.05 (*). A representative gel is shown in (c) which includes AVD-deficient samples (AVD) and control samples (Cont), n=8 per group.

Figure 5. Psychomimetic-induced hyperlocomotion in control and AVD-deficient C57BL/6J (a and c) and control and AVD-deficient BALB/c (b and d) mice in response to D-amphetamine (a and b), and MK-801 (c and d). The response to D-amphetamine was measured over 180 min and while there was a significant difference in hyperlocomotion between strains, there was no significant effect of Diet. The response to MK-801 was measured over 210 mins and there was no significant effect of Diet or Strain on hyperlocomotion, n=8-10 per group.

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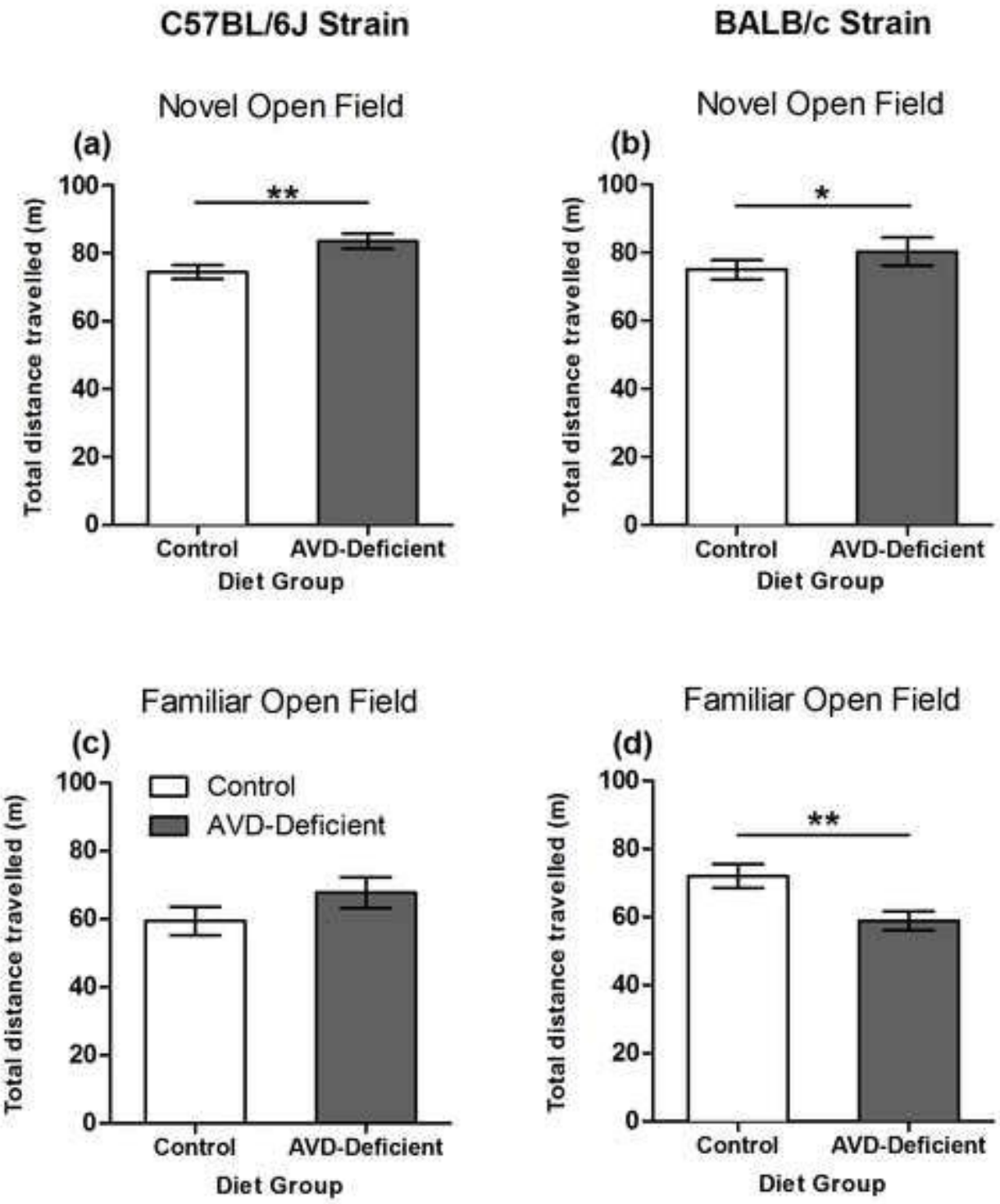

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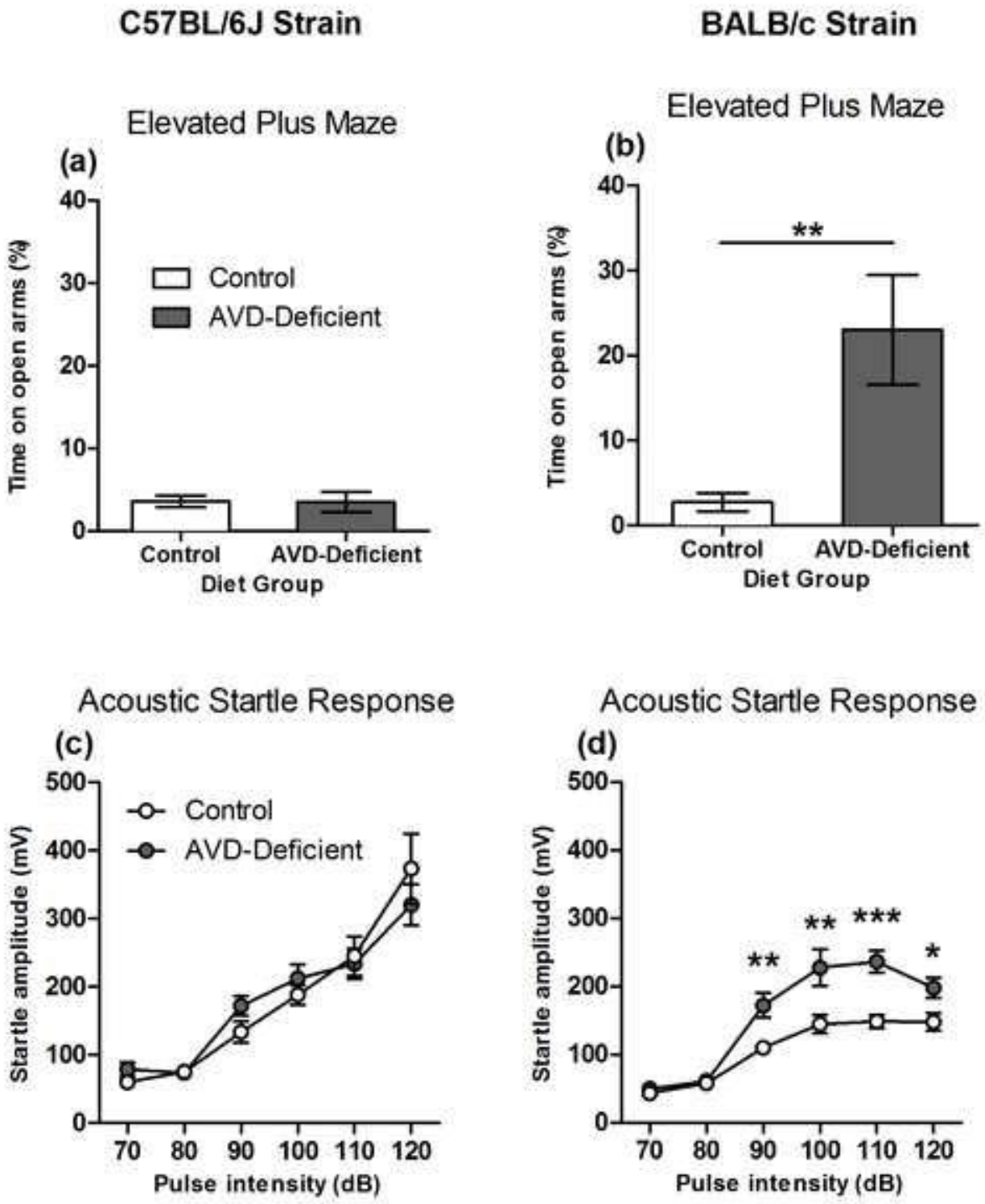

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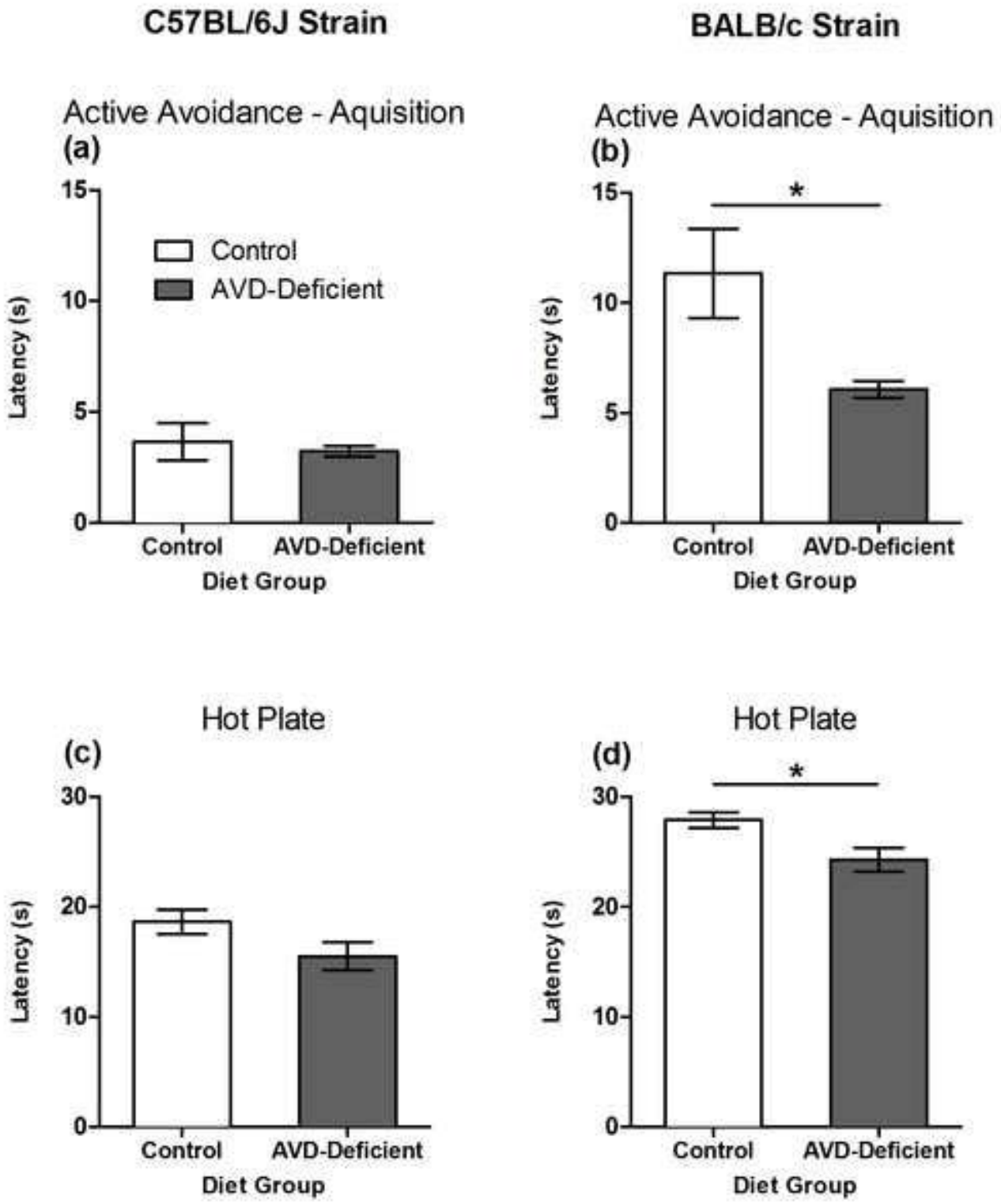

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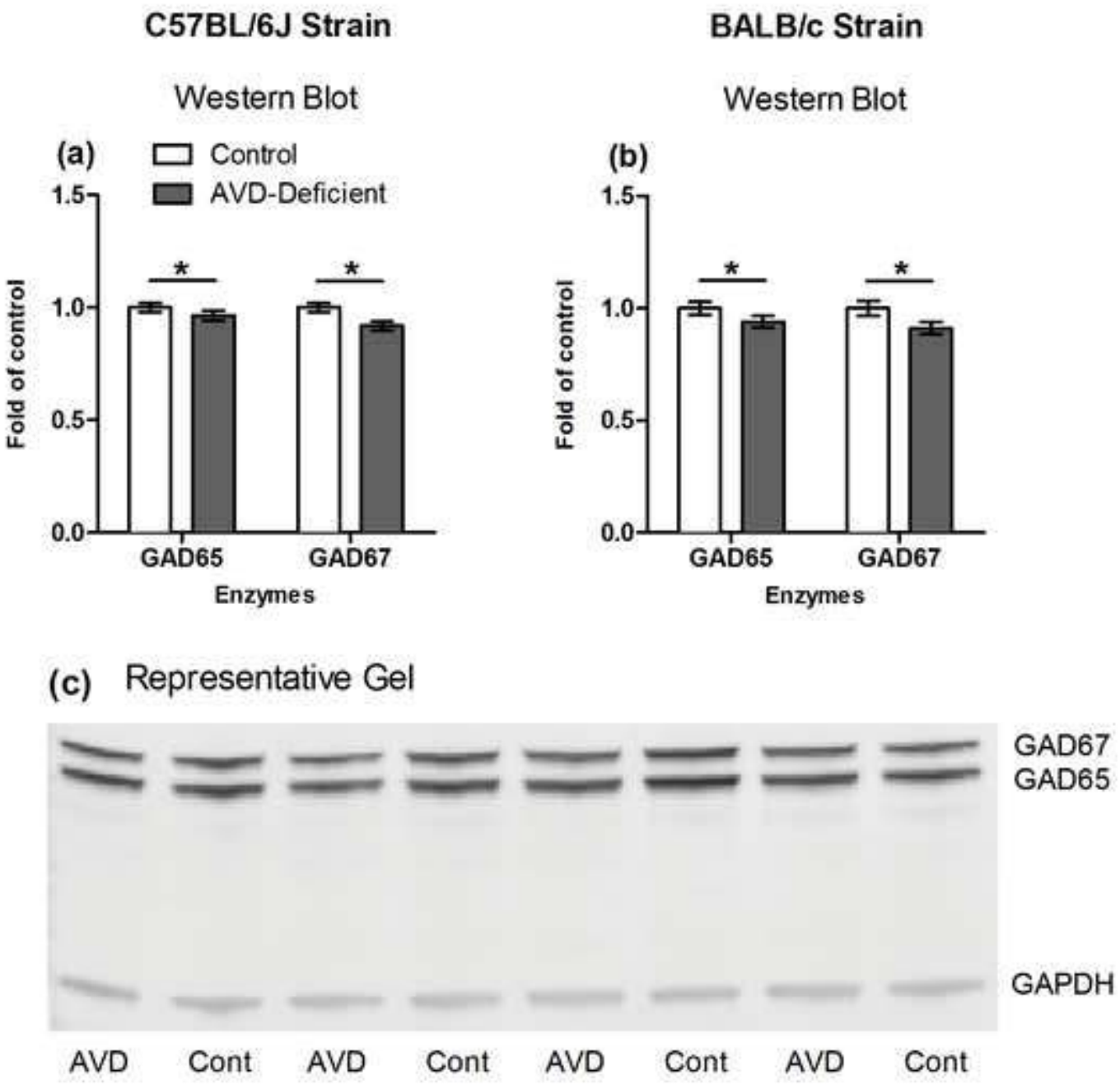

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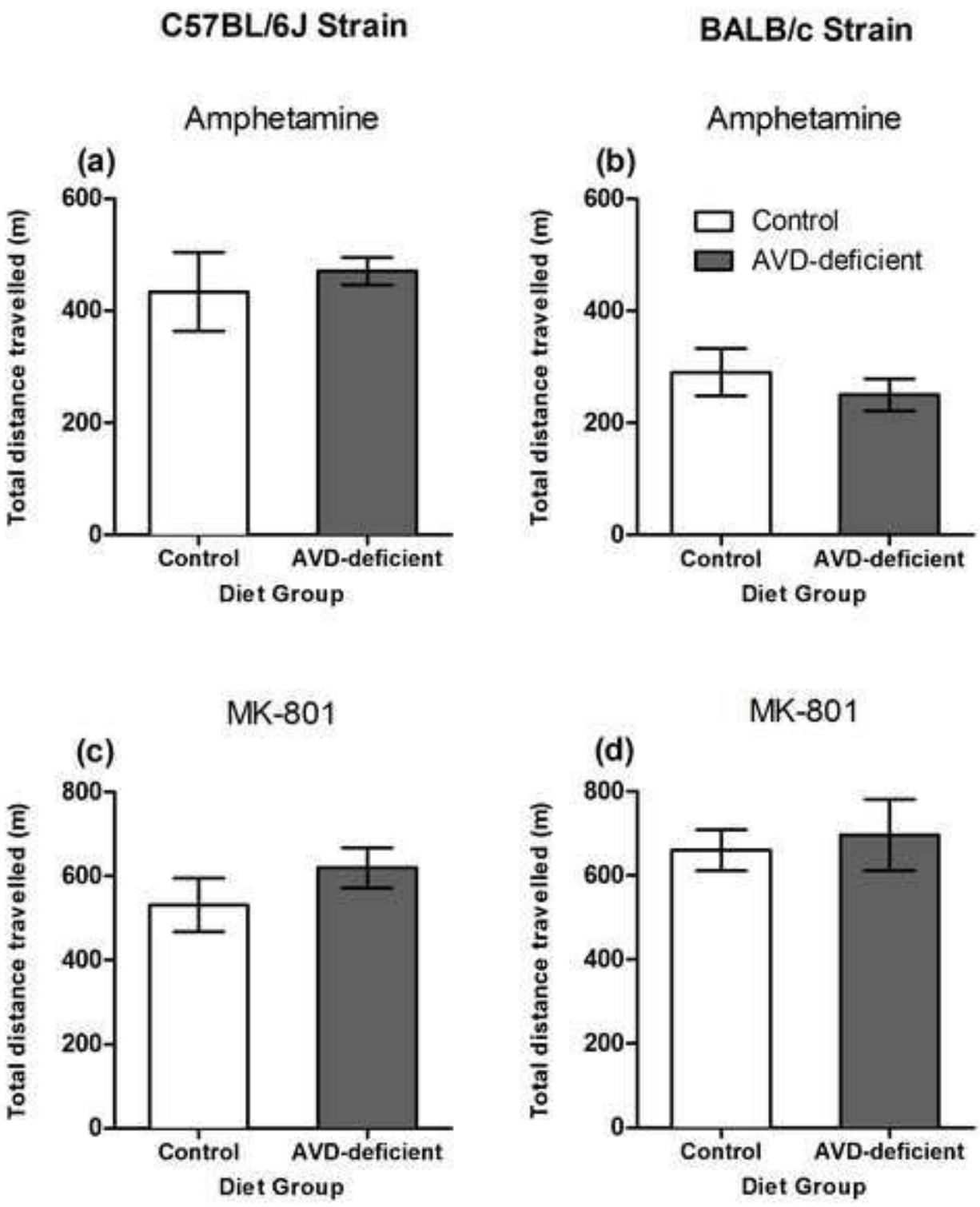

Table 1. Mean (± SEM) levels for catecholamines and amino acids in brain tissue of control and AVD-deficient C57BL/6J and BALB/c mice.

	C57BL/6J			BALB/c		
(ng/g)	**Control**	**AVD-deficient**		**Control**	**AVD-deficient**	
Catecholamines						
Noradrenaline	409.2 ± 4.7	387.6 ± 15.3		313.5 ± 18.8	349.2 ± 7.4	
Dihydroxyphenylacetic Acid	111.7 ± 2.0	111.7 ± 4.1		113.5 ± 2.2	120.1 ± 2.7	
Dopamine	1122.3 ± 23.5	1093.6 ± 27.1		1120.6 ± 16.1	1134.7 ± 18.4	
5-hydroxy-indoleacetic Acid	**329.2 ± 8.7**	**388.0 ± 13.8**	*	291.9 ± 13.9	311.3 ± 10.8	
Homovanillic Acid	**187.9 ± 4.3**	**207.7 ± 8.0**	*	197.3 ± 9.6	199.3 ± 5.9	
3-methoxytyramine	147.9 ± 5.2	150.6 ± 4.8		132.5 ± 4.7	132.3 ± 4.3	
5-hydroxytryptamine	557.1 ± 13.0	565.5 ± 9.1		507.7 ± 24.7	551.9 ± 12.3	
Amino acids						
Histidine	8.7 ± 1.7	12.2 ± 0.8		11.5 ± 2.3	15.2 ± 0.9	
Arginine	10.9 ± 1.5	10.7 ± 1.5		12.1 ± 0.9	13.6 ± 0.6	
Glutamine	659.9 ± 14.7	665.7 ± 56.2		**712.2 ± 27.6**	**642.4 ± 14.8**	*
Serine	**59.0 ± 0.8**	**67.7 ± 1.4**	*	50.3 ± 2.8	58.7 ± 2.9	
Aspartic Acid	330.7 ± 13.7	346.7 ± 9.6		292.3 ± 33.7	289.6 ± 31.8	
Glutamate	1287.3 ± 37.6	1280.2 ± 27.6		**1368.0 ± 39.1**	**1251.2 ± 22.6**	*
Glycine	54.6 ± 1.4	52.2 ± 1.3		**36.4 ± 0.7**	**38.9 ± 0.7**	*
Taurine	1229.0 ± 61.8	1108.3 ± 39.4		1102.5 ± 40.5	1108.3 ± 24.0	
Lysine	53.1 ± 9.5	48.7 ± 5.7		**38.9 ± 3.8**	**50.9 ± 2.6**	*
Tyrosine	14.1 ± 2.1	14.3 ± 2.0		6.5 ± 1.8	8.0 ± 2.0	
Alanine	28.3 ± 3.9	26.2 ± 5.6		26.8 ± 2.9	33.9 ± 5.0	
γ-aminobutryic Acid	273.2 ± 11.6	273.2 ± 4.5		**206.2 ± 5.4**	**223.9 ± 4.9**	*
Methionine	11.2 ± 0.3	10.9 ± 0.5		10.0 ± 0.4	10.0 ± 0.5	

* $P<0.05$ within strain comparison between control and AVD-deficient values.

Table 2. A summary of the significant results found in the two strains of AVD-deficient mice.

	C57BL/6J AVD-deficient	BALB/c AVD-deficient
Behavioural Test Battery		
Novel Open Field	△	△
Familiar Open Field	–	▼
Elevated Plus Maze	–	△
Acoustic Startle Response	–	△
Active Avoidance Aquisition	–	△
Hot Plate Test	–	△
Neurochemistry		
Glutamine	–	▼
Glutamate	–	▼
Glycine	–	△
γ-aminobutyric acid	–	△
5-hydroxyindoleacetic acid	△	–
Homovanillic acid	△	–
Serine	△	–
5-hydroxytryptamine: 5-hydroxy indoleacetic acid	▼	–
Dopamine: Homovanillic acid	▼	–
Proteins		
Glutamate decarboxylase 65/67	▼	▼

(△ increased, ▼ decreased, − no change). Experiments that had no change in either strain are omitted from this table (p = <0.05).